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Genetic predisposition to testicular cancer

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Chapter 7

Re-analysis of the Xq27-Xq28 region suggests a weak association of an X-linked gene with sporadic Testicular Germ Cell Tumour without cryptorchidism.

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Re-analysis of the Xq27-Xq28 region suggests a weak association of an X-linked gene with sporadic Testicular Germ Cell Tumour without cryptorchidism.

Introduction

The incidence of testicular germ cell tumour (TGCT) is still rising.⁽²⁶⁰⁾ Although the aetiology of TGCT is poorly understood, it is well known that male relatives (fathers/brothers/sons) of TGCT patients have an increased risk of developing TGCT. Currently 1-3% of TGCT patients report an affected relative.⁽²⁶¹⁾ Brothers of TGCT patients have an 8-10 fold increased risk of developing TGCT and the relative risk (RR) to fathers and sons is approximately 4-6.^(104;244) Because these RRs associated with an affected first-degree relative are considerably higher than for other cancers, which rarely exceed four, this observation most likely points to a genetic role in the aetiology of TGCT.⁽²⁴⁴⁾ Efforts have been made to identify TGCT predisposing genes. Although TGCT families have been reported in the literature, multigenerational pedigrees with several affected cases are rare and this limits the opportunities for linkage studies. In 1994, the International Testicular Cancer Linkage Consortium (ITCLC) was formed with the aim to collect TGCT families from all over the world and to perform genotyping studies. Recently, Rapley and colleagues (on behalf of the ITCLC) presented evidence for a TGCT susceptibility gene on chromosome Xq27.⁽¹⁰⁵⁾ Their genome-wide search for linkage in a set of 134 familial TGCT cases yielded a heterogeneity LOD (hlod) score of 2.01 on chromosome Xq27 using all families (n=99) compatible with X-linked inheritance and a hlod score of 4.7 on chromosome Xq27, if they included only families with at least one bilateral TGCT case (n=15) (genome wide significance level $p = 0.034$). In addition, 73% (n=14) of the familial TGCT cases with a history of cryptorchism (synonymous with cryptorchidism), a well known risk factor for TGCT, were linked to locus Xq27. These results provided evidence for a gene on chromosome Xq27 involved in TGCT susceptibility as well as in cryptorchism. Two recombination's, one between markers DXS8o43 and DXS8o28 on the centromeric side and one between FRAXA.pcr2 and FMR1Di on the telomeric side, bounded the identified TGCT1 locus, resulting in an interval of ~4 cM (~2.7 Mb). In this region, three genes have been reported so far: FMR1, responsible for Fragile X syndrome, a single exon gene Cxorf1 (expressed in the brain) and a tandemly duplicated gene LOC158813/158812.^(262;263) As yet, germline mutations in any gene in this region have yet to be identified as the cause of increased risk to develop TGCT.

An alternative approach to linkage studies is searching for TGCT susceptibility genes among unrelated TGCT cases in founder populations by means of association analyses on a dense set of markers. These so-called linkage disequilibrium fine-mapping analyses are based on the hypothesis that patients in founder populations inherited disease mutations from recent and common ancestors. A previous study showed geographic clustering of TGCT in the northern part of the Netherlands.^(62;95) Another study presented the results of analyses of HLA microsatellite markers and TGCT in this founder population.⁽⁹⁶⁾ The current study includes the previously used

study population, expanded with additional TGCT patients from the same founder population. The aim of this study was to corroborate or refute the previously observed linkage between TGCT and chromosome Xq27 using association analysis and the Haplotype Sharing Statistic (HSS).

Patients and Methods

Patients and controls

A total of 276 patients were randomly selected from all TGCT patients treated during the period 1977-2001 at the University Medical Center Groningen (UMCG), the Netherlands. The majority of these patients descended from three provinces (Groningen, Friesland, and Drente) in the Northern Netherlands, based on information collected about birthplace of the patient’s great-grandparents. Histological diagnosis was established for all patients by the department of Pathology of the UMCG.

Through the patients, family members (parents, children and spouse, brothers or sisters) were asked to participate. For this study on association of the X-chromosome with TGCT, only unaffected male first-degree family members were used and served as controls (n=169). Mothers of TGCT patients, if available, were used to check for correct inheritance. Population characteristics are presented in Table 1. TGCT cases were defined as familial cases when more than one TGCT case was present in the family. TGCT cases with male-to-male transmission (e.g. father – son) of the putative TGCT predisposition gene were excluded as these cases were obviously not compatible with X-linked inheritance and would have obscured the test results. The difference between the total number of non-seminomas (89%) and pure seminomas (11%) included is in particular due to different referral patterns for these histological subtypes. Traditionally all patients diagnosed with a non-seminoma within a defined area of the Comprehensive Cancer Centre in the northern part of the Netherlands (CCNN) are referred to the UMCG for further management after having been hemi-orchidectomised at the local hospital. In contrast, the majority of patients diagnosed

Table 1: Population characteristics	
	Number (%)
Patients	276
Non-seminoma	248 (89%)
Seminoma	28 (11%)
Bilateral TGCT	11 (4%)
Cryptorchism	43 (15%)
Familial TGCT	16 (6%)
Sporadic TGCT without cryptorchism	220 (79.7%)
Controls ^a	169
^a first degree, male family members	

with a seminoma are referred to one of the three radiation facilities within the CCNN area (including UMCG) for radiation treatment. All participants gave their informed consent and the Medical Ethical Committee of the UMCG approved the study.

Genotyping

High molecular weight genomic DNA was extracted from peripheral lymphocytes from 20 ml EDTA blood using standard protocols.⁽²⁵⁴⁾ After DNA extraction, a set of 16 polymorphic microsatellite markers in the Xq27-Xq28 region was genotyped in all patients and their relatives (see Table 2 for marker details, only markers that meet the quality criteria are shown, see section Results). Microsatellite markers were selected over a distance of approximately 4.3 Mb in order to cover the TGCT1 locus. Most markers were obtained from literature and the public databases. To get an evenly distributed set, the markers starting with XTC₀ were newly developed by searching the downloaded sequences for putative dinucleotide repeats and amplify these loci with primers selected with the online Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Relative marker positions were obtained from the contigs nt_011681.13 and nt_019686, and NCBI Map Viewer build 34 version 3 was used to determine the distance between both contigs. For each polymerase chain reaction (PCR), 0.25 units Taq DNA polymerase (Roche diagnostics, Mannheim, Germany) was used to amplify the fragments. The reaction volume was 10 µl. Reaction mixtures contained 200 µM of each dNTP, 1.5 mM MgCl₂, 10 mM tris-HCL, 50 mM KCL and 0.25 µM of each primer (with one primer 5’ labeled with a fluorochrome 6-FAM, HEX or NED). Cycling was performed on a PTC-225 thermal cycler (MJ research, Waltham, MA, USA). Amplification consisted of an initial denaturation of 5 min at 95°C, 35 cycles of 30 sec at 95°C, 30 sec at 55°C and 1 min at 72°C. Post PCR multiplexing was performed by combining 1-10 µl (based on signal strength) of PCR products. 2.3 µl of the pooled fragments was mixed with 2.5 µl deionised formamide and 0.2 µl ET-400R size standard (Amersham Pharmacia Biotech, Uppsala, Sweden) and separated on a MegaBACE 1000 capillary sequencer (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer’s protocol. Results were analyzed using Genetic Profiler v1.1 (Amersham Pharmacia Biotech, Uppsala, Sweden).

Statistical methods

Only markers that fulfilled the following quality criteria were included in the statistical analysis: failed genotyping (no results, alleles unknown) in less than 15% of the study population and erroneously scoring of two alleles in less than 4% of the male sub-population (men of course carry only one allele for each marker on their single X chromosome). In those few events where genotyping of an included marker erroneously revealed two alleles in a male, the genotype for that marker was set to “unknown”. All markers fulfilling the quality criteria were analyzed for allelic association by a chi-square test using only those alleles with an expected count of at least three. The data were also analyzed by the Haplotype Sharing Statistic (HSS).⁽²⁶⁴⁻²⁶⁷⁾ HSS is a linkage disequilibrium fine-mapping method that is based on the assumption that patients share disease mutations inherited from recent common ancestors. It measures the sharing between

Table 2: Characteristics of markers used for this Xq27 association screen (only markers that meet quality criteria are shown)

Marker	Relative position (Mb)	Primers		Failed PCR (%)	Heterozygous men (%)
		Forward	Reversed		
DXS8043	0.0000 ^a	AGTCTCAGAAACATTTGGTTAGGC	AATTATTGGCAAAGAGTACAGGCAG	6.5%	3.50%
DXS8028	0.21472 ^a	TGATGACACTCGGACTGC	GAAATAATAACTTGCCTTGCCT	13.7%	3.05%
AFMa1132f5	0.50108 ^a	AACACTGCACGATGAGAA	AGCTATCTGATTTGGTACT	4.6%	2.31%
DXS8045	1.48360 ^a	CAGGTAATCTGAGAAATGTTCTGC	ACTGCGGTGCTGACTAGG	7.5%	2.15%
DXS1200	1.71693 ^a	TACACACCAAAACACAGAGCCT	CTAGGGGCACCTTGAAAACAA	11.5%	2.30%
XTCo08	2.03909 ^a	TTCTGTCTCAAGCCAGATAA	CTGATCCTCTGACAGCATATAC	4.0%	3.21%
XTCo221	2.57815 ^a	TGTATCTGTGCATGTACCTATC	AAGAAGTCATCCACTGAGTCTA	2.2%	0.45%
DXS998	2.57935 ^a	CAGCAATTTTCAAAGGC	AGATCATTATATAACCTCAAAAGA	0.5%	2.20%
Fraxa1	2.95775 ^a	GATCTAATCAACATCTATAGACTTTATT	GATGAGAGTCACCTTGAAGCTGG	5.5%	0.70%
DXS1215	4.06973 ^b	GGGCAAAACATTAAACCTCTC	GCCCTCTAAGTCATTACGCT	4.4%	3.87%
DXS1193	2.95806 ^b	AATCTGACTCTGGGGC	TTATTTAAGGTGAGTATGGTGTGT	12.4%	1.27%
DXS1113	4.28679 ^b	GGGAGCTTTAGAGATTTTGGTAAAC	ACCTGTGGAGGATAGTAGTCTGACT	4.6%	3.20%

^a located on config nt_011681.13
^b location on config nt_019686, distance between contigs determined using NCBI Map Viewer build 34 version 3

a pair of haplotypes at a marker locus as the number of consecutive marker loci carrying the same alleles starting from the locus under analysis in both telomeric and centromeric direction. HSS then hypothesises that, in genomic regions containing disease mutations, haplotypes of patients show more sharing than haplotypes of controls. The association and HSS methods were also applied to four subgroups of patients, namely to patients with bilateral TGCT, to patients with familial TGCT, to patients with cryptorchism and to patients with sporadic TGCT without cryptorchism (Table 1). It should be noted that the number of patients with bilateral TGCT and the number of patients with familial TGCT were small.

Because multiple markers are analyzed, a multiple testing correction is required. This implicates that a result is only regarded as significant when the p-value is smaller than $0.05 / 12 = 0.004$, where 12 is the number of markers that passed our quality criteria. A 99.57% confidence interval corresponds to a 95% CI interval corrected for multiple testing. Power of the study was assessed by standard statistical theory on normally distributed variables, assuming that the allele counts follow a binomial distribution, which can be approximated by a normal distribution.

Results

Of the 16 microsatellite markers that were genotyped, four did not meet our quality criteria (data not shown). Hence, the analyses were performed on the 12 markers shown in Table2. Allelic association analysis did not reveal any significant difference, i.e. $p < 0.004$, between general TGCT patients and controls at any marker (Figure 1). In addition, the HSS did not show a significant result either (Figure 2). As the sample of Rapley and colleagues⁽¹⁰⁵⁾ consisted of familial TGCT cases and the linkage evidence became stronger when selecting only cases with bilateral TGCT or cases with cryptorchism, we also performed analyses on the subsets of patients. The results are shown in Figures 1 and 2. No significant associations were observed with any of these subgroups or any marker. We did however observe that for marker DXS1193 the major allele was less frequent among cases (88.3%) than among controls (96.6%) and that difference was smaller for cases with cryptorchism (94.7%) and those with familial TGCT (100%). Therefore, we also analysed the subgroup of sporadic cases without cryptorchism. In this subgroup, both allelic association and the HSS revealed evidence for a TGCT susceptibility locus ($p = 0.014$ and $p = 0.008$, respectively), which remain not significant after multiple testing correction (Table 3). However, after the multiple testing correction, a suggestive relative risk was observed for individuals carrying one of the minor allele at DXS1193 of 3.8 to develop TGCT and a significant relative risk of 4.7 (99.57% CI: 1.1-19.6) to develop sporadic TGCT without cryptorchism, compared with carriers of the major allele.

Table 3: Statistical results for association analyses on marker DXS1193

Marker	allele	controls	all	patients		sporadic, no cryptorchism
				Cryptorchism	familial	
DXS1193	107	96.6%	88.3%	94.7%	100%	86.1%
	105	0.0%	1.3%	0.0%	0.0%	1.7%
	109	0.0%	3.9%	2.6%	0.0%	4.4%
	110	2.7%	3.5%	2.6%	0.0%	3.9%
	112	0.7%	2.6%	0.0%	0.0%	3.3%
	114	0.0%	0.4%	0.0%	0.0%	0.6%
p-value			0.059	0.92	0.82	0.014
OR ^a			3.8	1.6	0.9	4.7
(99.57% CI)			(0.9-15.9)	(0.1-18.5)	(0.0-61.8)	(1.1-19.6)

^a OR (Odds Ratio) for combination of minor alleles

Discussion

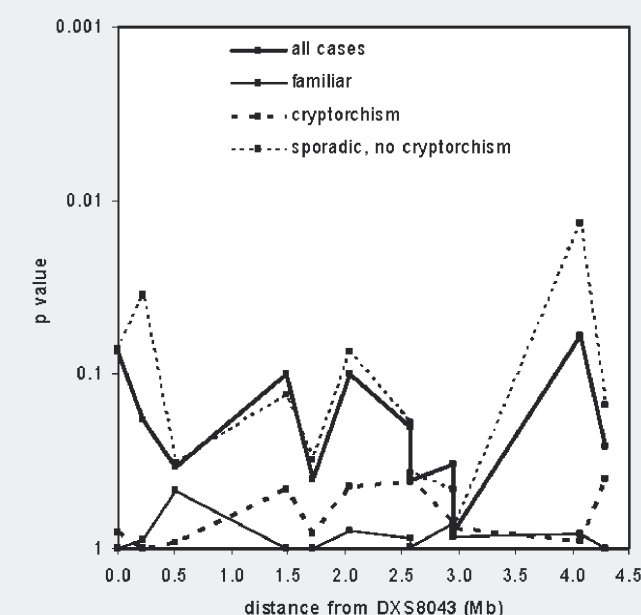
In the current study, we did not find an association between Xq27 and familial TGCT, cryptorchism or bilateral TGCT. We could therefore not confirm the results found by Rapley and colleagues.⁽¹⁰⁵⁾ We should however notice that our subgroups of familial or bilateral cases or cases with cryptorchism were small, which results in sufficient power only when a disease mutation with a frequency of 5% (or higher) and the putative gene has a large effect ($RR \geq 8$ for cryptorchism; $RR \geq 20$ for bilateral or familial cases). Hence, it cannot be excluded that an X-linked gene with a smaller effect is involved in familial or bilateral TGCT or cryptorchism.

Interestingly, we did observe an association between the subset of TGCT cases without a family history of TGCT or cryptorchism and specific alleles for the marker DXS1193 both by allelic association analysis and by the HSS. The frequency of all minor alleles was increased among these patients compared to controls: 13.9% versus 3.4%, respectively. The risk to develop sporadic TGCT without a cryptorchism for an individual who has one of the minor alleles was estimated to be 4.7 (99.57% CI: 1.1-19.6). This suggests that in our population one or more low frequent mutations of an Xq27-linked gene contribute to TGCT development but not to cryptorchism. Alternatively, particular genotypes in this region possibly protect the normal population from developing TGCT. Further analyses on single nucleotide polymorphism (SNPs) in candidate genes in this region should be performed to identify the causal gene and to unravel the nature of its causality.

Several observations had been made that could be interpreted as suggestive of the existence of a TGCT predisposing gene on the X chromosome. The increase in RR to brothers of TGCT

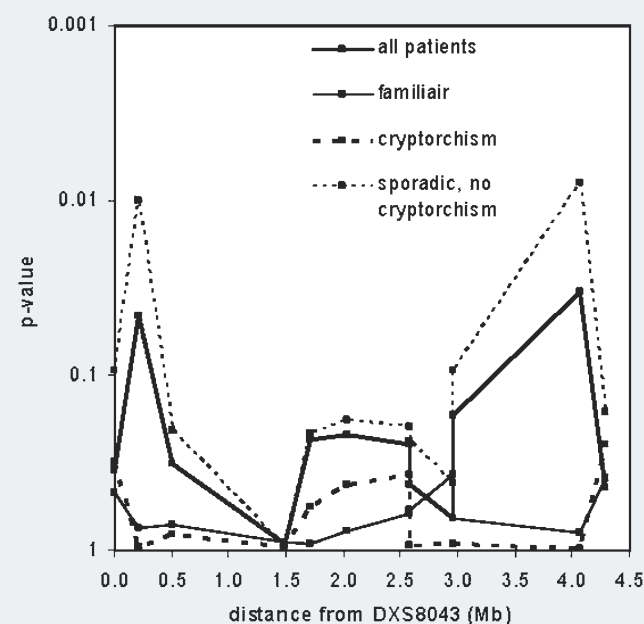
patients is higher than the increase in RR to fathers or sons of TGCT patients, which might be explained by assuming an X-linked inheritance of a TGCT predisposing trait. In addition, patients with Klinefelter syndrome (47, XXY constitutional karyotype) have a RR of 67 to develop mediastinal germ cell tumours. Like TGCT, these tumours are thought to arise from carcinoma in situ. The presence of an additional copy of the X-chromosome in Klinefelter syndrome suggests a possible dose-effect of one or more genes on this chromosome, escaping X-inactivation, on germ cell tumour development. Indeed, cytogenetic studies have revealed that generally the X-chromosome is overrepresented in TGCT tumour DNA.^(76;244;268) Ross and colleagues⁽²⁶⁹⁾ determined the sequence of over 99% of the gene-containing region of the X chromosome. They predicted that nearly 10% of the 1098 genes on the X chromosome are in a class that is upregulated in testicular and other cancers. Taken together, these observations suggest that the X chromosome may well harbour TGCT predisposing genes. As yet, these genes and their mutations remain to be identified.

Figure 1: Association analyses



Association analysis for alleles. The black squares represent the results at the markers. Lines between the markers are drawn only for an easier interpretation of the results. The lines distinguish the different (subgroup) analyses: a thick solid line for all patients, a thick dotted line for cases with cryptorchism, a thin solid line for the familial case and a thin dotted line for the cases without a family history of TGCT or cryptorchism. A p-value of < 0.004 is considered significant after multiple testing correction.

Figure 2: HSS



The Haplotype Sharing Statistic. The black squares depict the results at the markers. The different (subgroup) analyses are represented by different line styles: a thick solid line for all patients, a thin solid line for the cases with cryptorchism and a thin dashed line for the cases without a family history of TGCT or cryptorchism. The subgroup of bilateral cases was too small to perform a reliable haplotype sharing analysis. A p-value of < 0.004 is considered significant after multiple testing correction.

In a study by Rapley and colleagues a significant linkage was reported in 99 X-compatible pedigrees, in particular in families with cases with bilateral TGCT.⁽¹⁰⁵⁾ Recently Crockford and colleagues examined an additional 66 pedigrees with two or more cases of TGCT at Xq27. In contrast to the previous findings, they found no evidence for linkage at this region in this new set of pedigrees.⁽²⁶²⁾ Moreover three candidate genes from the identified minimal region at Xq27, FMR1, Cxorf1 and LOC58813/158812 were screened for small deletions, duplications and missense/non-sense mutations and no pathogenic mutations were observed. Crockford and colleagues also examined five genomic regions at four other chromosomes and for these regions no significant results were observed either.⁽²⁶²⁾ Their overall conclusion was that no single major locus can account for the majority of the familial TGCT cases. They suggest that multiple susceptibility loci with weak effects contribute to TGCT. Their conclusion is in line with our results. The region surrounding marker DX1193 could contain one of these susceptibility loci.

In conclusion, we could not confirm the previously reported association of familial, bilateral and cryptorchism-associated TGCT with Xq27, but we cannot exclude the presence of an X-linked gene that slightly or moderately increases risk to develop these particular phenotypes. Interestingly our data revealed an association between the subset of TGCT cases without a family history of TGCT or cryptorchism and marker DXS1193. Our findings suggest that in our population one but possibly more low frequent mutations of an Xq27-linked gene contribute to TGCT development but not to cryptorchism. It will be interesting to see whether these results can be confirmed in other populations. Until candidate genes from this region have been identified and can be checked for mutations, variations and their functional roles, the question of causal relation or statistical artefact remains unanswered.